AFRL-SR-AR-TR-09-0045

# REPORT DOCUMENTATION PAGE



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| 1. REPORT DATE *(DD-MM-YYYY)* | 2. REPORT TYPE | 3. DATES COVERED *(From - To)* |
|---|---|---|
| 30-01-2009 | Final | Feb 10, 2006 to Nov 8, 2008 |

**4. TITLE AND SUBTITLE**
Pharmacokinetic Manipulation and Modeling of the Trigger for JP-8-indueed Skin Irritation

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
FA9550-06-1-0173

**5c. PROGRAM ELEMENT NUMBER**

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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

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**8. PERFORMING ORGANIZATION REPORT NUMBER**

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AFOSR/NL
875 N Randolph St
Arlington, VA 22203
Dr. Walter Kozumbo

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION/AVAILABILITY STATEMENT**
Distribution A - Approved for public release

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

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**15. SUBJECT TERMS**
Jet fuel, skin irritation, skin penetration, gene expression, rats, human volunteers

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | 19b. TELEPHONE NUMBER *(Include area code)* |
| | | | | | |

# PHARMACOKINETIC MANIPULATION AND MODELING OF THE TRIGGER FOR JP-8-INDUCED SKIN IRRITATION

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Sponsored by AFOSR/NL

FINAL REPORT

30 January 2009

## ABSTRACT

Dermal exposure to JP-8 has been shown to cause skin irritation in humans and laboratory animals. We have previously investigated the early responses of mammalian skin to liquid JP-8 contact. We have developed a novel *in vivo* rat model for the investigation of skin irritation, which maintains the interactions and crosstalk of the wide variety of cells in the epidermis and dermis. We have used this model to investigate early gene expression with brief exposures to JP-8 and related skin irritants in both rats and humans. Our studies suggest that the "trigger" of the JP-8 irritant response may be a "physical" response that causes activation of signaling pathways that result in an inflammatory cytokine cascade as well as a competition between cell growth and apoptosis. We found that none of the four JP-8 components tested (2 aromatic and 2 aliphatic) exactly mimicked the JP-8 effects and that there were only minimal differences in gene expression between JP-8 and the synthetic fuel, S-8. Our similar studies in volunteers showed that the human variability was very large, but that the responses of the most sensitive volunteers were very similar to the rat responses.

The skin is a dynamic and intricate organ that is involved in many important physiological functions (temperature regulation, metabolism, mechanical support and protection as well as sensory functions) that may be interrupted by chemical irritation. We have been pioneers in the use of gene expression to study changes in signaling pathways and skin function as they are altered by chemical exposures. We have extensively investigated the time course of gene expression changed due to a 1-hour JP-8 exposure in the epidermis of the rat (McDougal *et al.* 2007c; McDougal and Garrett 2007b). Although there were never any visible signs of irritation after the one-hour exposure, Figure 1 shows that the number of genes changed two-fold continues to increase temporally after the beginning of exposure and that by 8 hours the number of genes decreased was larger than the number that was increased. The significant decrease in transcript level compared to control may reflect cellular damage, especially since the largest category of genes decreased by 8 hours was related to metabolic and physiological processes (McDougal *et al.* 2007b). According to gene ontology (generic GO slim) the nucleus was most affected in the cellular component category at the end of the exposure. More specifically, these were changes in the molecular functions involving binding of nucleic acid and DNA as well as transcription and translation regulator activity. Several signaling pathways related to inflammation, apoptosis, cell growth and proliferation were rapidly activated by JP-8 exposure. The very rapid response in gene expression is consistent with a "physical" stimulus due to interaction of the JP-8 with critical epidermal structures. JP-8 contains a mixture of aromatic and

aliphatic hydrocarbons with log octanol/water partition coefficients between 2.7 (toluene) and 7.6 (tridecane) that have been shown to penetrate into the skin (McDougal *et al.* 2000) and rapidly dissipate (Figure 2). Once in the epidermis, the JP-8 could move into the extracellular space between keratinocytes and partition into cellular, mitochondrial and nuclear membranes where they could initiate the stress responses that trigger signaling events. The interaction of JP-8 with membranes could be similar to the classic physical effect of ethanol on membranes (Goldstein 1984). From these results, we hypothesize that the "trigger" for the inflammatory process is "physical" stress involving JP-8 disruption of membrane integrity and the oxidative or osmotic balance which ultimately activates signaling pathways resulting in the recognized effects of JP-8 on the skin (Figure 3).

## BIOLOGICALLY-BASED PHARMACODYNAMIC MODEL

Based on the physical stress from JP-8 releasing interleukin-1α from cells in the epidermis, we have developed a kinetic model of the IL-1-stimulated intracellular signaling pathway in epidermal keratinocytes as an initial effort toward the pharmacodynamic modeling of JP-8 effects on the skin (McDougal *et al.* 2006). Upon exposure to external stimuli, such as chemical irritants, the skin secretes various cytokines and chemokines and evokes a cascade of events in the skin tissue. Therefore, the pharmacodynamic process of the skin primarily involves the responses of the skin cells to these endogenous proteins. Among them, one of central importance is IL-1, a proinflammatory cytokine that mediates

the host defense activities of the skin. The model captures the series of biochemical events initiated from IL-1α binding to IL-1 receptor (type I) on the cell surface that activates the transcriptional factor NF-κB and leads to production of a responsive protein, IL-6, as illustrated in Figure 4. The model takes into account two important autocrine regulatory loops of the system: the induction of a decoy IL-1 receptor (IL-1RII) and IL-1 receptor antagonist (IL-1Ra) by IL-1 signaling. The expressed IL-1RII and IL-1Ra compete with the normal receptor and ligands and serve as negative feedback regulations.

## COMPARISONS OF JP-8 WITH OTHER CHEMICALS

Based on the suggested differences in irritant characteristics that we found with xylene, SLS and limonene (Rogers *et al.* 2003), we compared the epidermal gene expression responses to two aromatic (dimethylnaphthalene (DMN) and trimethylbenzene (TMB)) and two aliphatic (undecane (UND) and tetradecane (TET)) chemicals with JP-8 responses. We wanted to determine if one of these components would mimic the gene expression response of JP-8 at the end of similar one-hour exposures. A secondary purpose was to compare the potency of these JP-8 components as neat chemicals in an attempt to determine if aliphatics or aromatics were responsible for the irritation induced by JP-8. Table 1 shows that the proportions of the selected aliphatics in JP-8 range from 6% (UND) to 1.8% (TET) and the proportion of the aromatics range from 1% (TMB) to 0.8% (DMN). Figure 5 confirms that the aliphatic chemicals have higher concentrations in the skin than the aromatic chemicals (not detected) at

the end of a JP-8 exposure (see Figure 2). The epidermal concentration of UND, TET, TMB and DMN in the skin when applied as a neat chemical showed no statistically significant differences. After analysis of gene expression, the number of genes changed two-fold and statistically significant for each treatment compared to a sham treatment are shown in Table 2. JP-8, UND and TMB caused changes in the largest number of genes followed by DMN and TET. One-way ANOVA Post Hoc analysis (Student-Neuman-Keuls) showed that the UND and TMB responses were similar, but JP-8, DMN and TET were all different. With each treatment, there were more genes decreased than increased, ranging from 57% (UND) to 78% (TMB) of the total genes changed. UND and TMB caused the greatest number of gene changes, more than twice as many as DMN, and about ten-fold more than TET. According to Ingenuity Pathways Analysis (IPA), the top functions affected by JP-8 were cell death, cellular growth and proliferation and cellular movement. Table 3 shows that UND and TMB most closely mimicked the number of genes related to cell damage, growth & proliferation, and cellular movement changed by JP-8. Figure 6 demonstrates that none of the component exposures exactly mimicked the biological processes changed by JP-8. Chemical-induced changes in gene expression with the four components showed consistent differences in magnitude, whether total gene expression or functional pathway-specific changes were investigated. When only the genes related to specific pathways or functions changed by JP-8 were considered, we found that these pathways were nearly all activated by the components, but to different extents. Analysis of

chemical concentrations in the skin after the component exposures indicated that the nearly 10-fold differences in gene expression response (Table 2) were not due to different target tissue (epidermis) concentrations (Figure 7). UND and TMB appear to be more potent inducers of gene expression in the epidermis than DMN and TET, but both aliphatic and aromatic compounds cause responses that may result in irritation. As far as we know, this study was the first attempt to relate *in vivo* changes in gene expression to tissue concentrations. We concluded that no single component will mimic the response of the complex JP-8 mixture, but in our study UND had the most similar responses. This study (McDougal and Garrett 2007a) does not allow us to state that either aromatics or aliphatics are responsible for JP-8-induced skin irritation.

The USAF is considering replacing some JP-8 with a synthetic fuel (S-8), which doesn't contain the aromatic hydrocarbons, and is made from coal, natural gas or biomass using the Fischer-Tropsch process. We have investigated the changes in epidermal gene expression after brief, 1-hour, exposures to S-8 or JP-8 using our standard exposure model and were unable to find dramatic changes in epidermal mRNA levels at the end of the exposure (McDougal, unpublished results). Of the 29,740 gene transcripts that were identified in the skin, 1,147 of the S-8 samples had expression levels that were significantly different from the JP-8 samples with 4 skin samples in each treatment group. At the significance level of P=0.05, 1,487 differences would be expected by chance alone, suggesting that the differences were not real. Almost none of these genes were recognizable as genes we have previously seen altered in epidermis with

chemical treatments. When the Benjamini-Hochberg multiple testing correction was used there were no statistically significant differences at the P=0.05 level. Figure 8 shows that of all the genes that were marked as changed due to S-8 treatment without the Benjamini-Hochberg multiple testing correction, only 3 transcripts had differences that were greater than 2 fold increased with S-8 compared to JP-8. These transcripts were the cytokine CCL2, heat shock protein 1b (HSPA1b) and pleckstrin (PLEK). The cytokine responds strongly to irritating chemicals on the skin (McDougal *et al.* 2007a) and a two-fold difference probably is not a significant event. This heat shock protein and pleckstrin are not commonly affected in irritation. We conclude that with our brief exposures model to rat skin, there are no dramatic differences due to S-8 and JP-8 treatments.

In an attempt to compare the gene expression effects of repeated JP-8 exposures with two other irritants in our *in vivo* rat model, we investigated 10-minute JP-8 exposures, once a day for 6 days, with identical exposures to octane (a minor component of JP-8) and cumene (an irritant) (McDougal et al., *in preparation*). Table 4 shows that the magnitude of gene expression changes due to JP-8 was about 6-fold less than that of octane and cumene. Visual observations and histological analysis of skin sections indicate that the superficial and microscopic damage from JP-8 was less than the other chemicals (data not shown). Figure 9 shows that most of the genes changed by repeated JP-8 exposures (84%) were also changed by the other irritants. According to IPA 6.5, four of the top five biological functions changed by these treatments were similar – Cellular movement, Cell death, Cell-to-cell signaling and interaction, Cellular

growth and proliferation. The only unique biological functions were Post-translational modification for JP-8, Cell signaling for octane, and Lipid metabolism for cumene. In addition, Table 5 shows the relative magnitude (fold-change) of changes for representative genes related to irritation for JP-8 were less than those modulated by exposure to octane and cumene. These results suggest that JP-8 and the other irritants cause quantitative differences in responses of the skin, but qualitatively the changes are very similar. These quantitative differences relate both to the number of transcripts significantly changed more than the two-fold threshold and to the magnitude of the changes when we compare individual genes across the treatments.

## BRIEF EXPOSURE STUDIES WITH HUMAN VOLUNTEERS

We studied changes in gene expression due to brief (1-hour) JP-8 cutaneous exposures in humans (McDougal et al., *in preparation*) over the same time course (up to 8 hours) as previously studied in rats (McDougal *et al.* 2007d). After approval by the appropriate institutional review boards, 12 adult male volunteers (ages 18 to 57) were divided into 3 groups and exposed to 0.5 ml JP-8 in a Hilltop chamber on the surface of one buttock. The other buttock was sham-treated with an empty chamber. The chambers were held in place with surgical tape and the individuals were allowed to move around normally during the exposure. All treatment groups had the chambers removed after one hour, the site wiped with ethanol on a gauze pad, and one 8 mm biopsy punch was taken from each site under local anesthesia 1, 4 and 8 hrs after the beginning of

the exposure. The epidermis was separated from the rest of the skin with a cryotome and total mRNA was isolated. Gene expression was measured with standard Affymetrix microarray (HG-U133 plus 2) techniques and changes from sham treatment were analyzed with GeneSpring GX 9.0 Expression Analysis and Ingenuity Pathway Analysis 5.5.1. After appropriate quality control, probe sets with expression levels below 20 percentile were removed from the analysis leaving 49,027 of the 54,645 available probe sets on the microarray chip.

The type of transcripts that are constitutively expressed in human skin can provide information about the cutaneous functions and the ability of the skin to respond to insults. When the sham-treated skin samples from all twelve subjects were analyzed using IPA, there were 38,401 transcripts present in the epidermis and 37,922 transcripts present in the dermis. Of these, 10,036 and 9,993 were mapped to the IPA knowledge base for epidermis and dermis, respectively. In both the epidermis and dermis, the top five functional categories that these transcripts belonged to were cell death, cell growth and proliferation, gene expression, cell cycle and post-translational modification. This is in contrast to the results we found in rat skin, five years earlier (Rogers *et al.* 2003) in which metabolism, oxidative stress and signal transduction were the most prevalent genes with the Affymetrix Rat Toxicology U34 array; however, this array only contained 850 genes. Since then, Affymetrix arrays (rat and human) have covered the complete genome and give better representations of the normal functions involved in the skin.

In these human exposure samples, individual variability was at least as great as the responses to JP-8 treatment, because the two-way ANOVA revealed that there were no differences between the treatments (sham & JP-8), or between the post-exposure times (1, 4 or 8) and no interactions between treatment and post-exposure time at P=0.05. Unlike the recent rat studies (McDougal *et al.* 2007e) where the same treatments and times could be grouped together and averaged, the human variability in gene expression responses was so high that each individual had to serve as his own control and the gene expression data was reanalyzed and uploaded to IPA. Figure 10 shows the number of genes for each individual that were changed 2-fold by JP-8 treatment compared to sham. Three individuals (2 sampled at 4 hours and 1 sampled at 8 hours) showed changes in gene expression that were much greater in magnitude than the rest of the individuals providing evidence for the popular notion of "sensitive skin" in some individuals. Because of variable sensitivity and testing of each individual at only one time point, we cannot conclude that four hours after the beginning of the exposures is the time of maximum response. Figure 11 shows that the responses of the sensitive volunteers had 900 genes in common, which were similar to many of these genes and the functions and pathways they affect were also changed in the rat studies. Four of the top five functions consistently altered in the rat studies (McDougal et al., *in preparation*) were also consistently altered in the sensitive volunteers (Table 6). The exceptions were transcripts related to the gene expression function, which was not in the top five

in the rat and cell cycle function transcripts which were not in the top five in the humans.

Many studies have investigated interleukin 8 (IL-8) release from human keratinocyte cultures in response to JP-8 dosing (Allen *et al.* 2000; Allen *et al.* 2001a; Allen *et al.* 2001b; Inman *et al.* 2008; Monteiro-Riviere *et al.* 2004; Yang *et al.* 2006). IL-8 is a proinflammatory cytokine, found in humans and mice but not rats, which has been shown to be one of the major mediators of irritant responses. Figure 12 shows some genes related to IL-8 that were changed in each responsive individual. IL-8 mRNA was upregulated by about 17- to 27-fold in the three responders; none of the non-responders had increases in IL-8 transcripts over the control levels. IL-8 is a CXC chemokine that is one of the major mediators of the inflammatory response, where it functions as a chemoattractant (Sticherling *et al.* 1991) and a potent angiogenic factor (Rosenkilde and Schwartz 2004). Vascular endothelial growth factor (VEGFA), which was also upregulated (4.2- 6.0-fold), has been shown to increase the expression of IL-8 mRNA in endothelial cells (Lee *et al.* 2002). Both beta 1 integrin (ITGB1) and alpha 6 integrin (ITGA6) proteins found in plasma membranes were also upregulated and as a complex have been shown to be involved in VEGF expression (Chung *et al.* 2004). The mRNA for prostaglandin-endoperoxide synthase 2 (PTGS2), which is involved in cell growth (Murakami *et al.* 2000) was increased about 7-fold. Transcript levels were increased in several signaling molecules, including eukaryotic translation initiation factor 4E (EIF4E), cyclin-dependent kinase inhibitor 1A or p21 (CDKN1A), SMAD family member 3

(SMAD3), and v-myc myleocytomatosis viral oncogene homolog (MYC). Transcripts for the nuclear receptor (NR3C1) were decreased more than 3-fold which might be expected to decrease cellular proliferation, but there is conflicting evidence in knockout mice that reduction of this protein can increase proliferation (Wintermantel *et al.* 2005). These changes are consistent with the stimulation of epithelial cell growth by brief JP-8 exposure as part of the homeostatic response. Our studies in human volunteers confirm the importance of IL-8 in the inflammatory responses to JP-8, which have only previously been shown in human keratinocyte cultures. These *in vivo* studies suggest that IL-8 release is a very good measure of the potential for an irritant response. In general, it appears that the brief exposure *in vivo* rat skin model may be a good surrogate for the gene expression responses of "sensitive" individuals. These studies confirm that many of the functional responses and signaling pathways are similarly affected by irritation of the rat and human epidermis.

## CONCLUSIONS

Changes in gene expression levels are expected to provide information about the adaptive and toxic responses to any exogenous hydrocarbons that enter the skin, because the mRNA levels generally precede production of new proteins in the skin, particularly those related to metabolism, oxidative and cellular stress and signal transduction. Microarray studies provide the ability to survey tens of thousands of gene products in one experiment and are very useful for elucidating patterns of response and generating hypothesis that can assist the

understanding of the mechanism of any deleterious effects. Our studies with brief cutaneous exposures in rats suggest that the "trigger" of the irritant response may be a "physical" response, which disrupts cellular or mitochondrial membranes. The primary proinflammatory cytokine (IL-1α) released from this process can be modeled using a biologically based pharmacokinetic model to describe the changes in protein levels. Changes in gene expression in response to JP-8 suggest the activation of signaling pathways that result in the inflammatory response as well as both apoptosis and cell growth and proliferation. We have found that the response of the four components of JP-8 that were compared with the JP-8 gene expression response in the epidermis were different than that of the complete mixture, and that no single component (or aromatic or aliphatic classification) mimicked the JP-8 effects, although undecane was most similar. From our research, the differences in gene expression between S-8 (the Fischer-Tropsch prepared fuel) were not dramatic. We did find that repeated exposures to JP-8 were much less irritating than similar exposures to cumene or octane. When we repeated the JP-8 skin exposure study that we accomplished in rats in human volunteers we found that there was tremendous variability in the human epidermal response. We confirmed, in our *in vivo* model, that *in vitro* assays which measure interleukin-8 release as a measure of irritation are valid. When the responses of the most affected volunteers were compared with the rat responses we saw that the rats were good models for the gene expression responses of the "sensitive" humans.

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**Table 1. Composition of USAF-provided JP-8 sample as analyzed by gas chromatography. (McDougal et al., 2000)**

| Component | Percent (w/w) |
|---|---|
| Undecane | 6.0 |
| Dodecane | 4.5 |
| Decane | 3.8 |
| Tridecane | 2.7 |
| Tetradecane | 1.8 |
| Methyl naphthalenes | 1.2 |
| Nonane | 1.1 |
| Trimethyl benzene | 1.0 |
| Pentadecane | 1.0 |
| Dimethyl naphthalenes | 0.78 |
| Dimethyl benzene (xylene) | 0.59 |
| Naphthalene | 0.26 |
| Ethyl benzene | 0.15 |
| Diethylene glycolmonomethyl ether | 0.08 |
| Methyl benzene | 0.06 |

**Table 2. Number of transcripts changed Two-fold and statistically significant ($P \leq 0.05$) in each cutaneous treatment compared to sham. (McDougal and Garrett, 2007)**

| | JP-8 | TMB | UND | DMN | TET |
|---|---|---|---|---|---|
| Increased | 476 | 289 | 683 | 207 | 44 |
| Decreased | 635 | 1,018 | 523 | 263 | 110 |
| Total | 1,100 | 1,307 | 1,206 | 470 | 154 |

**Table 3. Number of transeripts changed by each of the treatments that were related to the top 3 functions changed by JP-8. Indented eategories are subcategories of the one above and their numbers are ineluded in the eategory above. (MeDougal and Garrett, 2007)**

| | JP-8 | UND | TMB | DMN | TET |
|---|---|---|---|---|---|
| Cell Death | 212 | 210 | 197 | 88 | 40 |
| Apoptosis | 169 | 170 | 153 | 71 | 35 |
| Survival of eukaryotic cells | 62 | 65 | 59 | 29 | NS |
| Cellular Growth & Proliferation | 228 | 238 | 232 | 98 | 45 |
| Growth | 153 | 157 | 156 | 66 | 39 |
| Proliferation | 160 | 92 | 150 | 65 | 31 |
| Proliferation of keratinocytes | 8 | 7 | 6 | 6 | NS |
| Cellular Movement | 128 | 141 | 105 | 55 | 34 |
| Chemotaxis | 46 | 47 | 16 | 19 | 17 |
| Rolling of eukaryotic cells | ns | 7 | NS | NS | NS |

NS = none significantly changed

**Table 4. Biologically (2-fold) and statistically significant (P≤0.05) changes in gene expression after six 10-minute cutaneous exposures compared to control.**

| | JP-8 | Octane | Cumene |
|---|---|---|---|
| Upregulated | 79 | 424 | 439 |
| Downregulated | 47 | 325 | 324 |
| Total | 126 | 749 | 763 |

**Table 5. Magnitude of fold changes for some irritant-related genes upregulated by the JP-8, octane and cumene treatments for 6 days.**

| | JP-8 | Octane | Cumene |
|---|---|---|---|
| CXCL1 | 35.6 | 97.1 | 115.8 |
| MMP13 | 18.3 | 29.3 | 30.8 |
| CXCL3 | 9.3 | 26.6 | 42.2 |
| S100A9 | 7.8 | 21.9 | 16.3 |
| PTSG2 | 7.4 | 18.7 | 21.3 |
| IL-6 | 4.9 | 16.9 | 23.7 |

**Table 6. Cellular and Molecular Functions of the genes changed in each responsive individual. Mean number of genes changed by the JP-8 treatment is listed.**

| Function | P-value range | No. of genes (mean) |
|---|---|---|
| cellular growth and proliferation | $1.8x10^{-19} - 3.3x10^{-4}$ | 554 |
| cell death | $8.2x10^{-19} - 4.7x10^{-4}$ | 487 |
| cell cycle | $1.4x10^{-13} - 4.4x10^{-4}$ | 242 |
| cellular development | $8.1x10^{-13} - 4.2x10^{-4}$ | 393 |
| cellular movement | $1.1x10^{-9} - 4.7x10^{-4}$ | 294 |

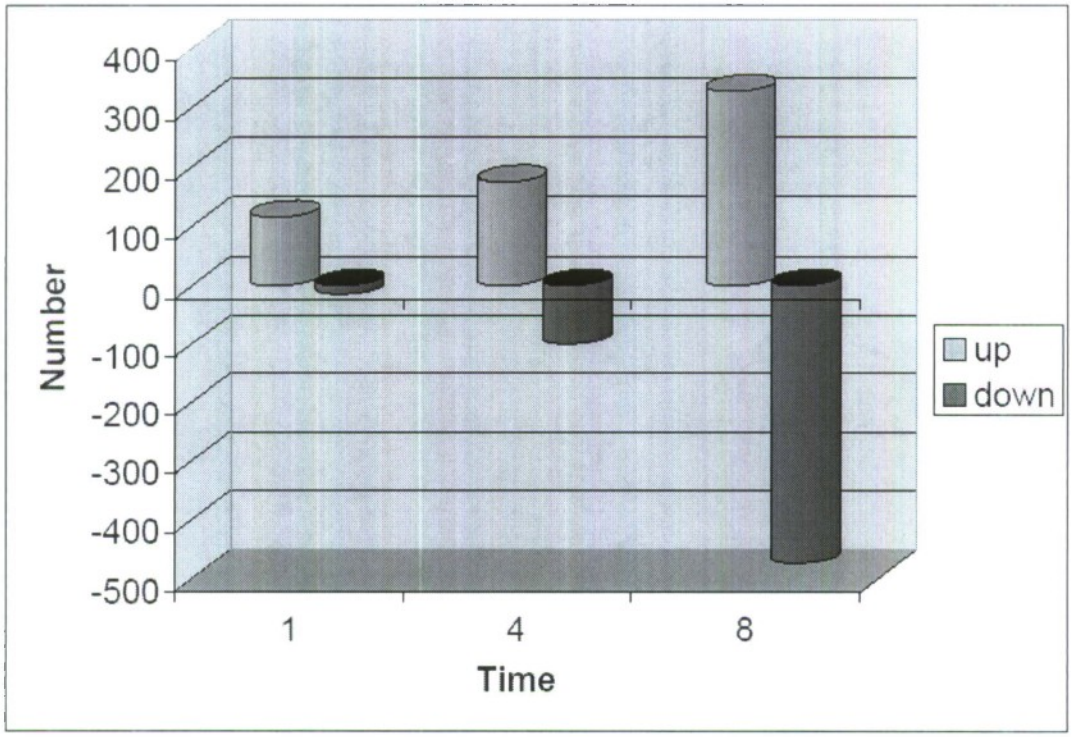


Figure 1. Plot of the number of genes changed two-fold with time in the epidermis after the beginning of the 1-hour JP-8 exposure to the rat. (McDougal et al., 2007)

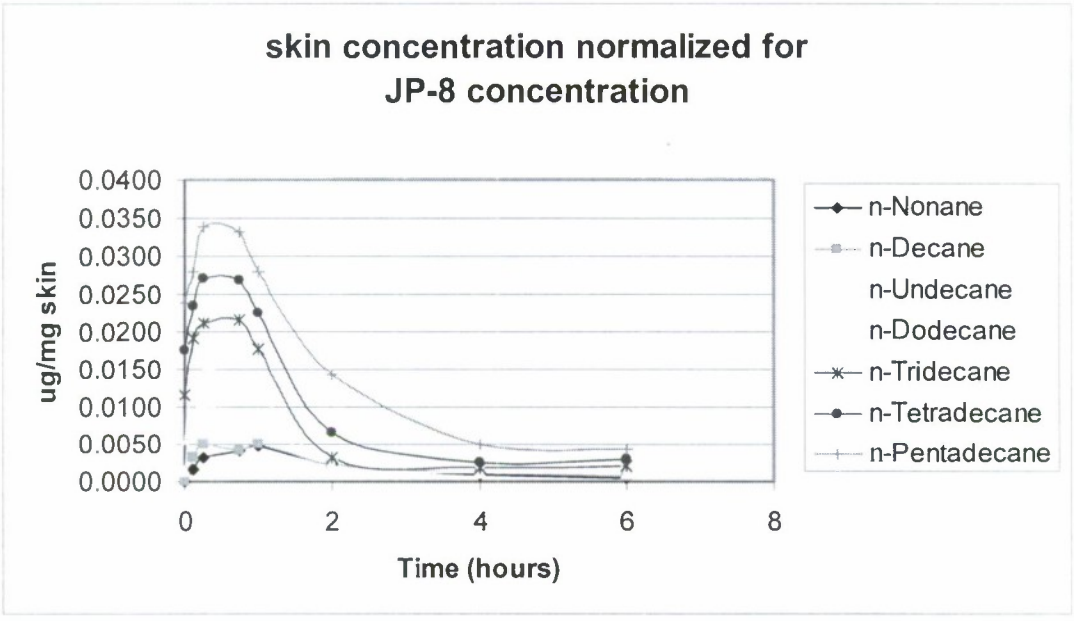


Figure 2. Concentration of seven JP-8 components in rat skin after a 1-hour *in vivo* exposure to JP-8. The concentrations were normalized for the concentration of each component in JP-8.

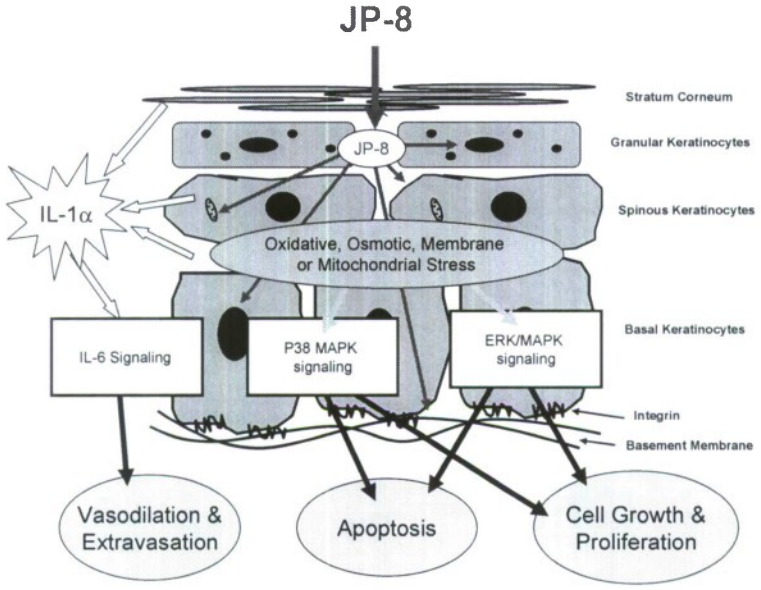


Figure 3. Schematic of the proposed mechanism of "physical" damage in the epidermis caused by JP-8 exposure. The stress response involves increases in signaling pathways that result in inflammation, apoptosis, growth and proliferation. (McDougal et al., 2007)

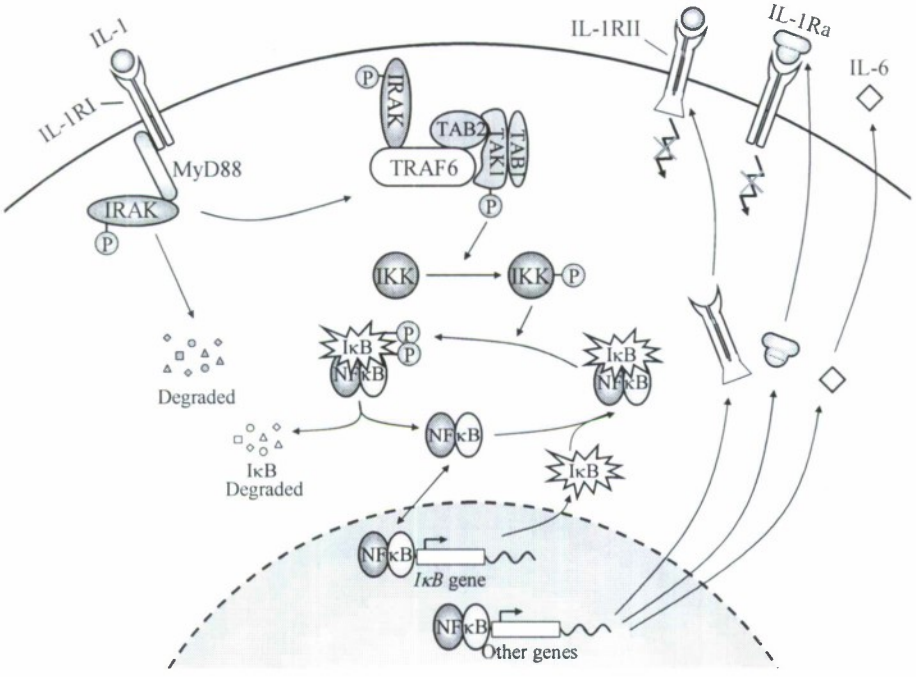


Figure 4. The major biochemical events of the IL-1 signaling pathway in keratinocytes. Receptors on the cell surface are shown at the top with the associated ligands and the nucleus is at the bottom. (McDougal et al., 2006)

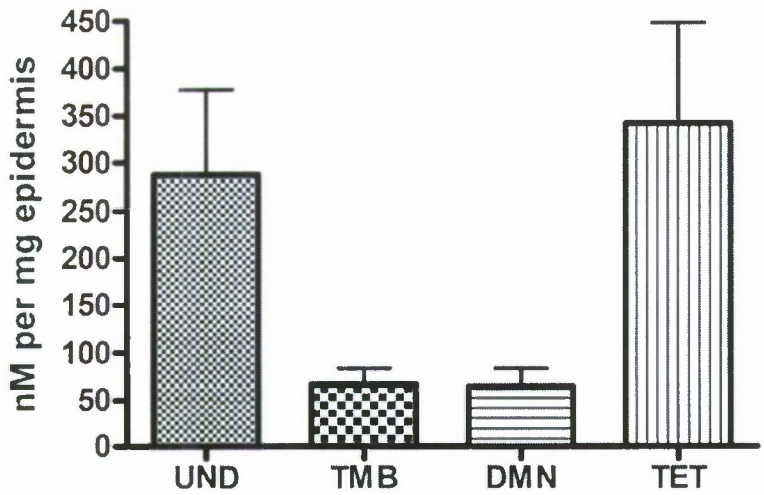


Figure 5. The concentration in the epidermis of four components measured by gas chromatography at the end of a 1-hour JP-8 exposure. $P \leq 0.001$ with ANOVA, N=5. UND = undecane, TMB = trimethylbenzene, DMN = dimethylnaphthalene, and TET = tetradecane. (McDougal and Garrett, 2007)

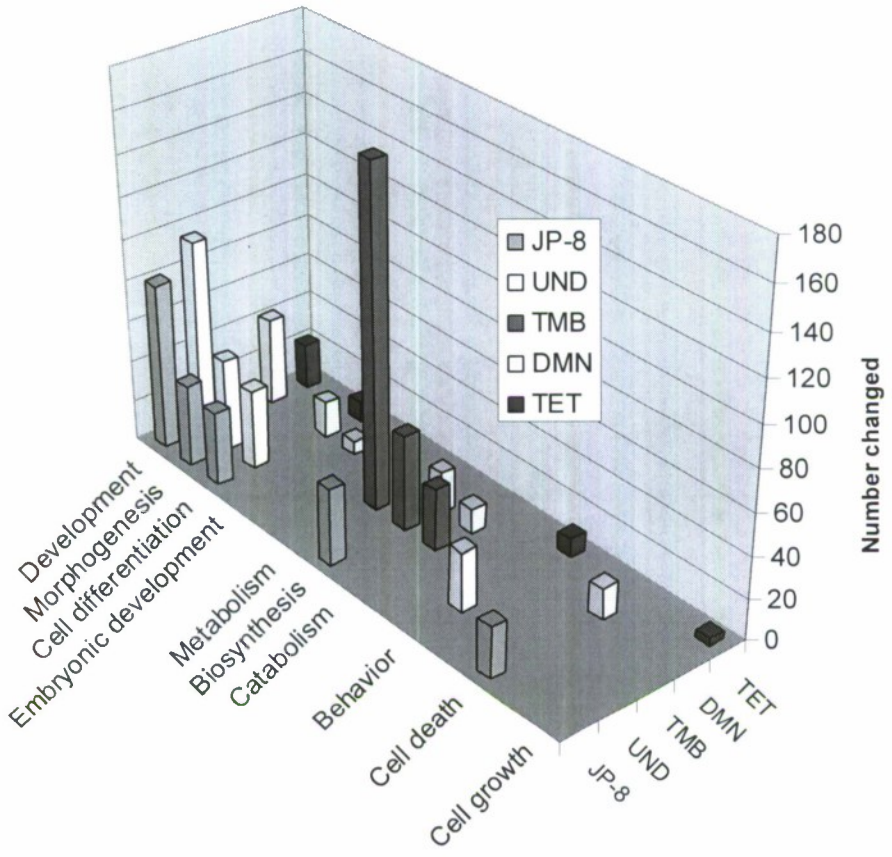


Figure 6. Graph of Gene Ontology Biological processes with numbers of transcripts changed for each of the treatments. UND = undecane, TMB = trimethylbenzene, DMN = dimethylnaphthalene, and TET = tetradecane. (McDougal and Garrett, 2007)

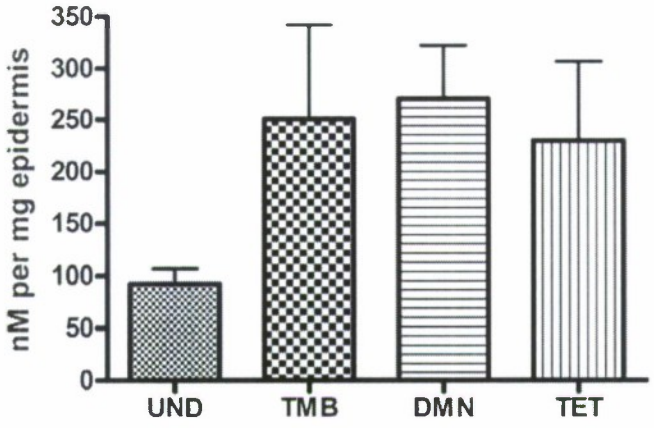


Figure 7. The epidermal concentrations of the four components applied as pure chemicals as measured by gas chromatography at the end of 1-hr exposures. UND = undecane, TMB = trimethylbenzene, DMN = dimethylnaphthalene, and TET = tetradecane. $P \leq 0.25$ with ANOVA. N=5 (McDougal and Garrett, 2007)

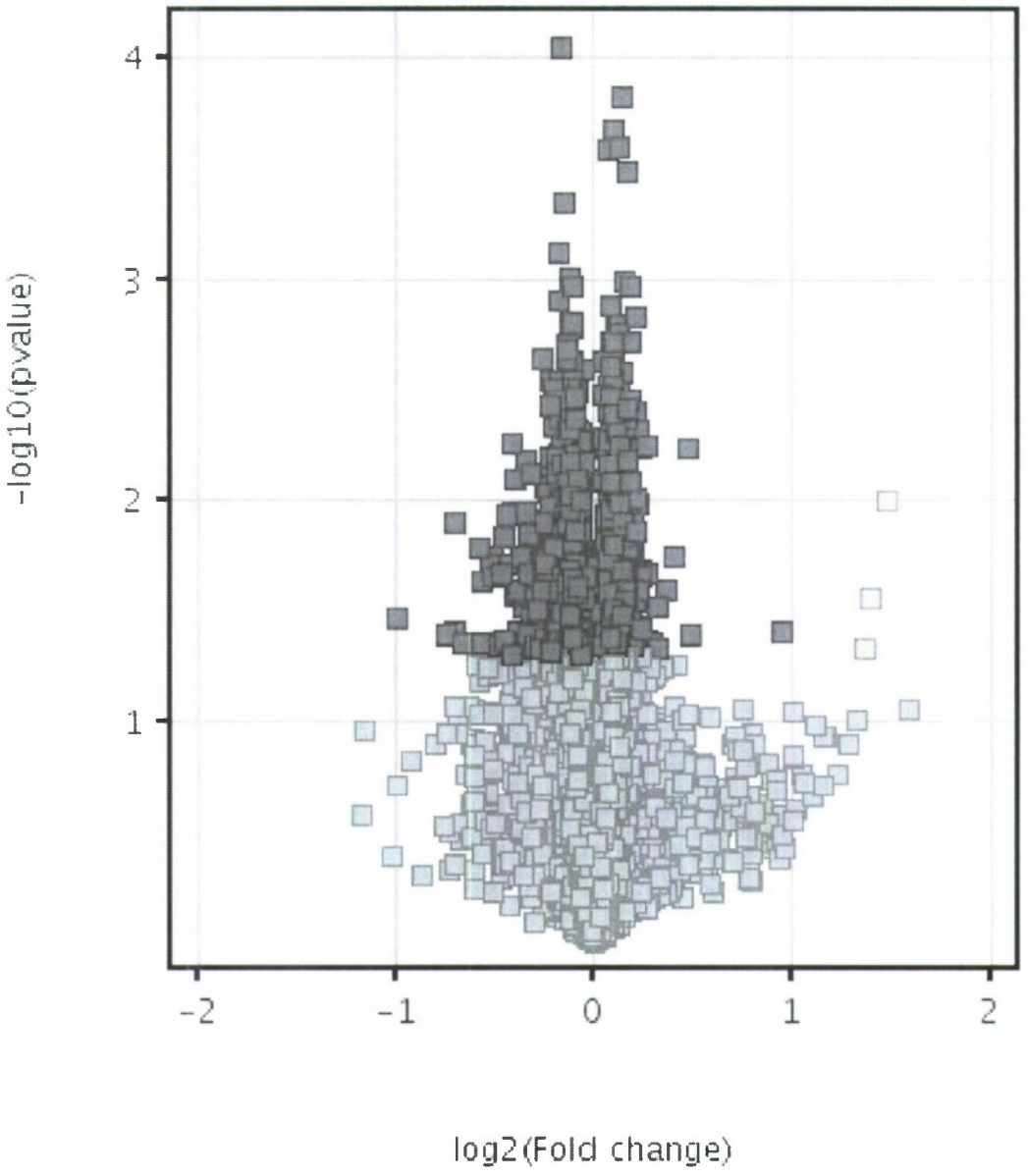


Figure 8. Volcano plot of the comparison of expression of individual genes between S-8 and JP-8 treatments. The transcripts marked in red have an P value of 0.05 or greater (uncorrected for multiple tests) and the ones marked in green have both a P value of 0.05 or greater and two-fold differences.

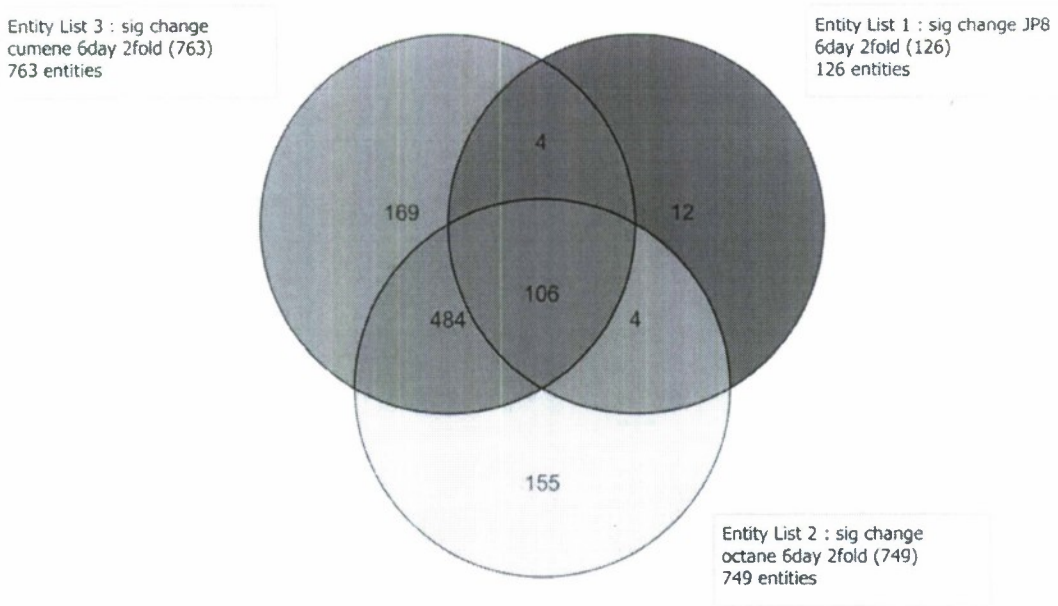


Figure 9. Venn diagram of the changes in gene expression with the three chemical treatments, showing numbers of common and unique transcript changes.

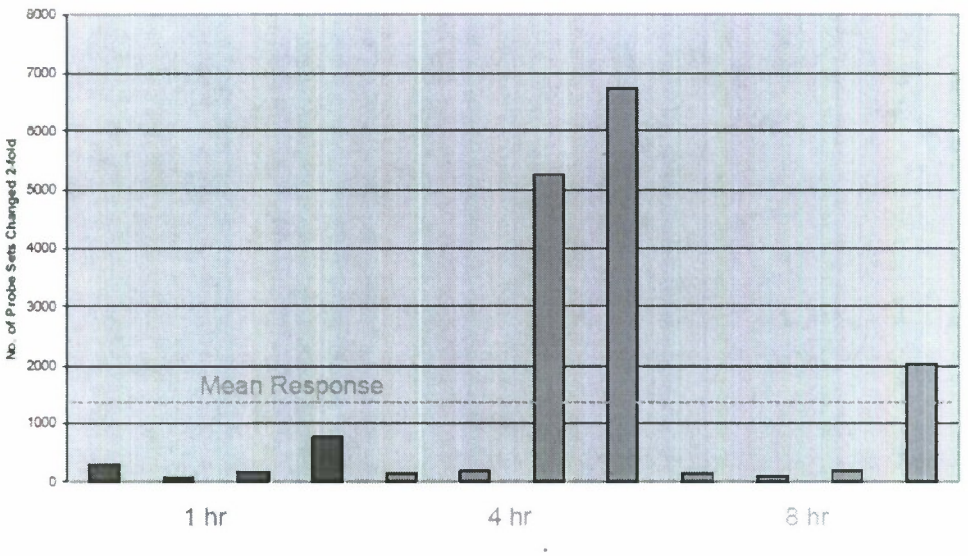


Figure 10. Number of probe sets altered two-fold in the individual subjects. Mean response is shown as a dotted line. Colors indicate time after the beginning of the one-hour JP-8 exposure.

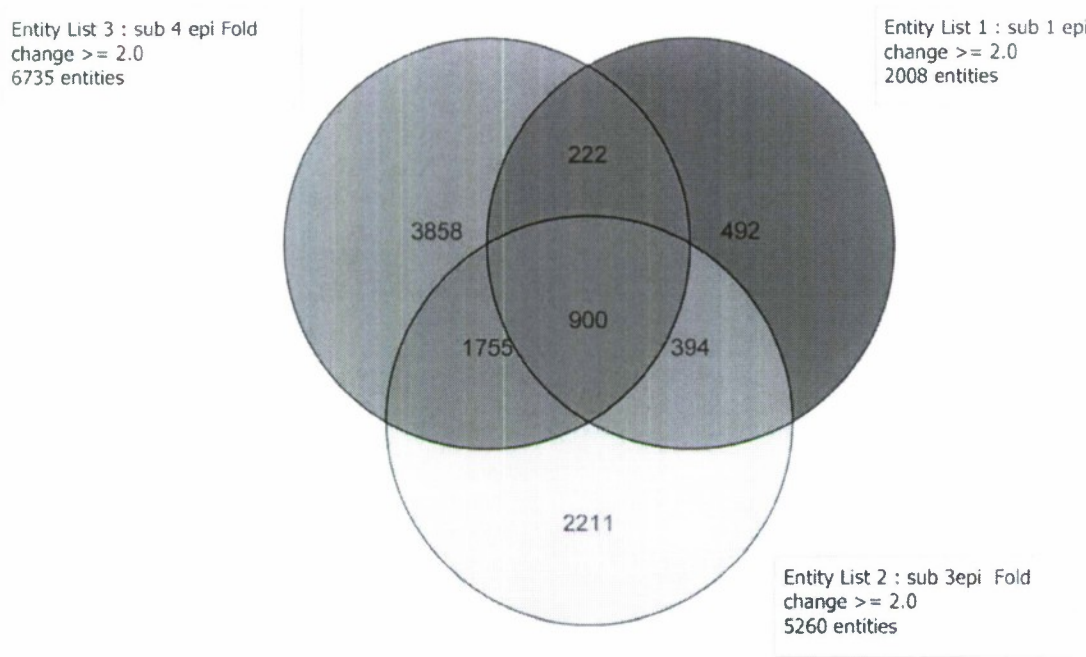


Figure 11. Venn diagram of the changes in gene expression for each responsive volunteer showing numbers of common and unique transcript changes.

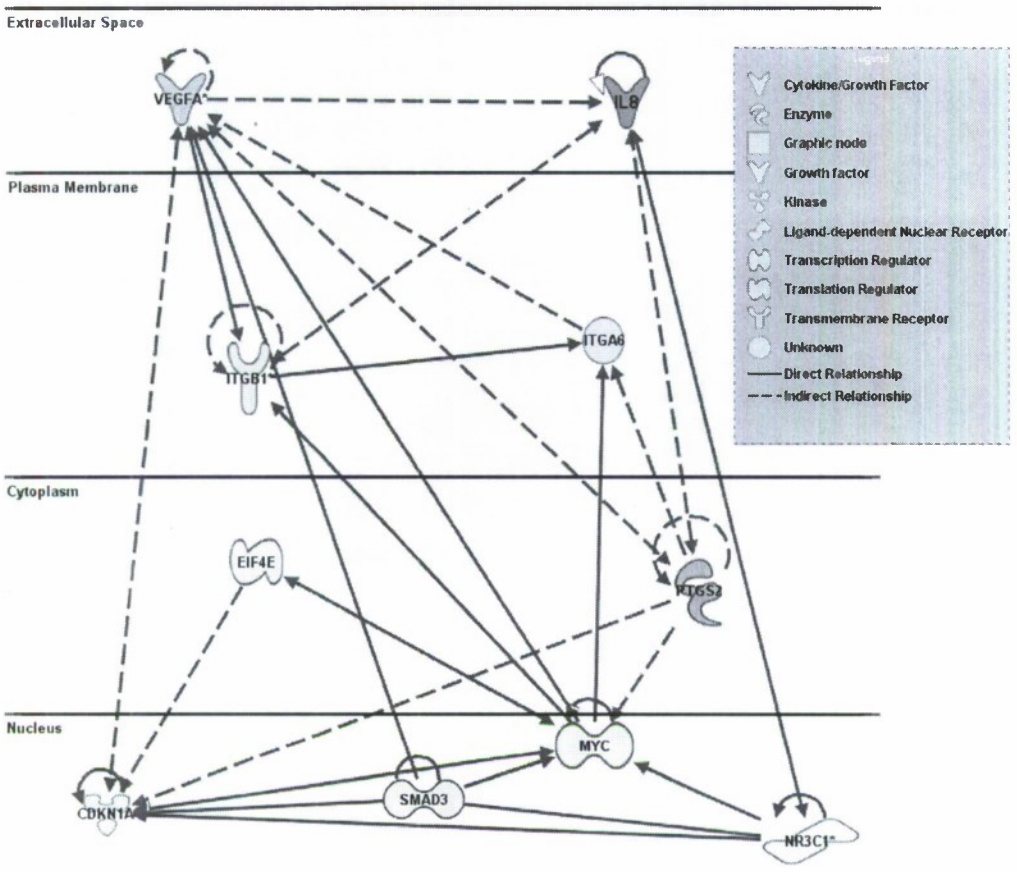


Figure 12. Genes related to proliferation of epithelial cells that were changed in each responsive individual. Genes colored red were up-regulated and genes down-regulated were colored green. The intensity of the color related to the level of up- and down-regulation of subject 4. See text for abbreviations.